UNIVERSIDADE DE LISBOA
FACULDADE DE CIÊNCIAS

DEPARTAMENTO DE BIOLOGIA ANIMAL

EPIGENETIC MODIFICATIONS IN THE OFFSPRING AFTER SPERMATOGONIAL STEM CELL TRANSPLANTATION

Sara Maria Cabrita de Rezende Mântua Mota

DISSERTAÇÃO

MESTRADO EM BIOLOGIA HUMANA E AMBIENTE

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VRIJE UNIVERSITEIT BRUSSEL
FACULTEIET GENEESKUNDE EN FARMACIE

BIOLOGY OF THE TESTIS

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Abstract

Background: Mouse spermatogonial stem cell transplantation (SSCT) has become an established research model to study the testicular germ cell line. By performing SSCT in mice, donor spermatogenesis can be re-established in the seminiferous tubules of an otherwise infertile recipient. Transplanted males are able to produce fertile offspring after spontaneous mating.

Therefore, SSCT is a promising fertility preservation technique for prepubertal boys who are exposed to gonadotoxic treatments, leading to infertility.

Compared to the increasing reports illustrating the effectiveness of SSCT in reproductive terms, only a few studies addressed safety issues. Before accepting SSCT as a clinical strategy, all potential safety concerns need to be carefully evaluated.

In the present project we investigated whether epigenetic modifications occur in the correct way during early embryogenesis and during spermatogenesis in offspring.

Material & Methods: We evaluated the general methylation level, using immunohistochemistry for 5-methylcytosin (5-MC), in pre-implantation embryos of different stages (2-cell, 4-cell, multi-cell, morula and blastocyst) from GFP^- transplanted mice with GFP^+ testicular donor cells. We also analyzed the acetylation of H4K8.

Stainings for 5-MC, DNMT3a, H4K5ac and H4K8ac were also performed on testicular tissue from live born GFP^+ male offspring of transplanted mice.

Results: Unfortunately, no GFP^+ embryos could be obtained from the transplanted males. The GFP^+ offspring showed a correct pattern for H4K5ac and H4K8ac in the spermatids, but some abnormalities were seen in the spermatocytes. No major differences were indicated for DNMT3a and 5-MC compared to fertile adult controls.

Conclusion: No major abnormalities on epigenetic level were observed, and the ones observed do not seem to cause anything severe. Anyway, it is still necessary to repeat the embryo experiment to check possible epigenetic problems during embryo development. Therefore, even though SSCT has potential as a clinical application, more research is required before becoming a fertility preservation technique.

Keywords: Epigenetic modifications; Fertility preservation; Pre-implantation embryos; Spermatogenesis; Spermatogonial Stem Cell Transplantation (SSCT)

Sumário

Quinze anos após a sua introdução, o transplante de células espermatogoniais estaminais (SSCT) no rato tornou-se um modelo de investigação estabelecido para o estudo e manipulação da linhagem germinativa das células testiculares. Através do SSCT em ratos, a espermatogénese de um dador é reestabelecida nos túbulos seminíferos de um receptor infértil. Machos transplantados são capazes de produzir descendência após acasalamento espontâneo, e também foi demonstrada fertilidade nos descendentes.

Portanto SSCT é uma técnica promissora para a preservação da fertilidade de rapazes que ainda não atingiram a puberdade e que são expostos a tratamentos gonadotóxicos que provocaram uma eventual infertilidade.

No entanto, apesar das evidências que ilustram a eficácia do SSCT em termos reprodutivos, apenas alguns estudos abordam questões de segurança ao nível de modificações da linha germinativa, como, por exemplo, aberrações cromossómicas, alterações no imprinting e modificações epigenéticas. Por isso, antes do SSCT ser aceite como estratégia clínica, todos os potenciais problemas devem ser verificados e avaliados.

Neste projecto, vamos investigar se algumas modificações epigenéticas ocorrem ou não de forma correcta durante a embriogénese e durante a espermatogénese dos descendentes de ratos transplantados.

Foi avaliado o nível de metilação geral, fazendo himuno-histoquímica para o 5-metilcitosina (5-MC), em embriões de diferentes estádios de desenvolvimento embrionário (2-células, 4-células, multi-células, morula e blastocisto) de ratos GFP^- transplantados com células testiculares de uma dador GFP^+. Também foi avaliada a aceptilação da H4K8.

Colorações para 5-MC, DNMT3a, H4K5ac e H4K8ac foram realizadas em tecido testicular de descendência GFP^+ nascida dos ratos transplantados.

Infelizmente não se conseguiram obter embriões GFP^+ a partir dos machos transplantados. A prole GFP^+ nascida mostrou um padrão correto para a aceptilação da H4K5 e da H4K8 nos espermatídios, mas algumas alterações foram observadas nos espermatócitos. Não foram detectadas grandes diferenças para o DNMT3a e a 5-MC em relação aos controlos adultos férteis.

Ou seja, não foram observadas grandes alterações ao nível epigenético, e as observadas parecem não causar nada de grave. De qualquer forma, ainda é necessário repetir a experiência ao nível do desenvolvimento embrionário para se verificar possíveis problemas epigenéticos durante o desenvolvimento do embrião. Portanto, embora SSCT tenha um grande potencial para a aplicação clínica, é necessária mais investigação antes de se tornar uma técnica de preservação da fertilidade.

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List of Abbreviations

5-MC: 5- methylcytosine

A_{al}: $A_{aligned}$ spermatogonia

A_{pr}: A_{paired} spermatogonia

A_s : A_{single} spermatogonia

BSA: bovine serum albumin

DNAse: deoxyribonuclease

DNMTs: DNA methyltransferases

EDTA: ethylenediaminetetraacetic acid

FCS: Fetal calf serum

FACS: Fluorescent-Activated Cell Sorting

MACS: Magnetic-Activated Cell Sorting

NGS: normal goat serum

GDNF: glial cell line-derived neurotropic factor

GFP: green fluorescent protein

hCG: human chorionic gonadotropin

H4K5ac: histone 4 lysine 5 acetylation

H4K8ac: histone 4 lysine 8 acetylation

H4K12ac: histone 4 lysine 12 acetylation

H4K16ac: histone 4 lysine 16 acetylation

PBS: phosphate buffered saline

PMSG: pregnant mare's serum gonadotropin

PS: pachytene spermatocytes

RS: round spermatids

Spc: spermatocyte

Spg: spermatogonia

Spt: spermatid

SSCs: spermatogonial stem cells

SSCT: spermatogonial stem cell transplantation

1. Introduction

1. Introduction

1.1 Male reproductive system

The male reproductive system consists of a penis, two testes (gonads) with epididymis, deferent duct and the accessory glands. The testes are the focal point of the male reproductive system as they produce the male gametes, the spermatozoa, in a process known as spermatogenesis.

The testes have two main functions: production of sperm necessary for sexual reproduction and production of male sex hormones necessary for functional development of the sex organs. The testes are located together with the epididymis in the scrotum and are surrounded by a membranous layer, the tunica albuginea, and are further divided into different lobules. Each lobule is composed of different seminiferous tubules, in which spermatogenesis occurs (Junqueira and Carneiro, 2004).

The male germ and Sertoli cells are located in the seminiferous tubules, while the Leydig cells (responsible for the production of testosterone after puberty) are located in the interstitium. Seminiferous tubules are delineated by the basal membrane, which is surrounded by one or several layers of myeloid cells (Roberts, 2010).

All seminiferous tubules drain to the rete testis from which the spermatozoa are transported through the efferent ducts to the epididymis, where the spermatozoa are stored and undergo a process of maturation. The deferent duct transports the sperm through the genital glands to the penis during ejaculation (Junqueira and Carneiro, 2004; Roberts, 2010) (Figure 1).

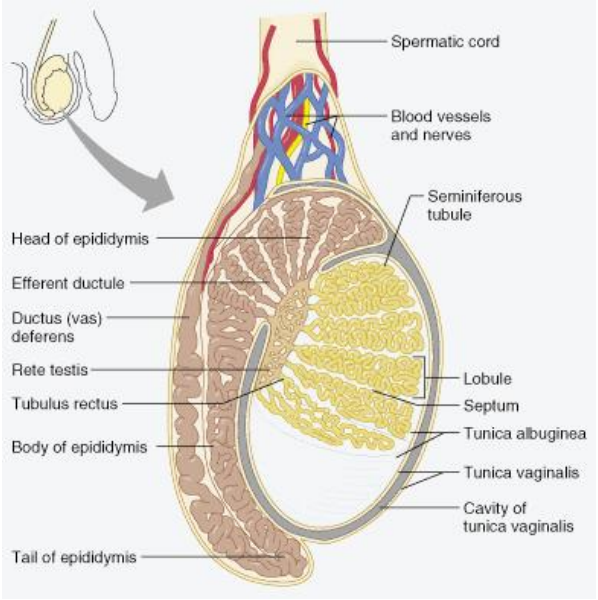

Figure 1. *Anatomy of the human testis (http://legacy.owensboro.kctcs.edu/gcaplan/anat2/notes/APIINotes2%20male%20reproductive%20anatomy.htm)*

1.2 Spermatogenesis

Spermatogenesis is a strictly regulated and continuous process. Every day, millions of spermatozoa are produced in the normal adult testes.

Spermatogenesis is supported by a stem cell population, the spermatogonial stem cells (SSCs), which are situated on the basement membrane. Differentiation occurs from the basement membrane towards the lumen (Goossens and Tournaye, 2013) and depends on hormonal and cellular signaling, which begins at puberty and is maintained throughout life (McLean, 2005; Dym *et al.*, 2009).

SSCs are crucial for spermatogenesis and thus male fertility. Although these cells are highly important, they are very scarce. In the adult rodent testis only 0.03% of all germ cells are considered to be a true stem cell (Phillips *et al.*, 2010). Like other stem cells, SSCs can replenish the stem cell pool by mitotic divisions (self-renewal) or they can initiate differentiation in order to produce spermatozoa (Kubota and Brinster, 2006).

1.2.1 Spermatogonial stem cell niche

The behaviour of SSCs in terms of self-renewal and differentiation must be strictly regulated to prevent SSC exhaustion because of too much differentiation, or an overproduction of SSCs. To regulate and control the balance between self-renewal and differentiation, SSCs reside within a specialized microenvironment, called the niche, which regulates stem cell behaviour. A stem cell niche can be described as an area in which the stem cell resides and is maintained throughout life. The SSC niche is composed of adjacent differentiated cells, Sertoli cells, peritubular myoid cells, interstitial Leydig cells and the extracellular matrix components (Figure 2) (de Rooij, 2009; Hwang and Lamb, 2010; Caires *et al*., 2010; Kolasa *et al*, 2011).

Sertoli cells play an important role in the regulation of SSC behaviour and spermatogenesis, because they produce a number of growth factors, e.g. glial cell line-derived neurotropic factor (GDNF), fibroblast growth factor 2 and stem cell factor that have an effect on SSCs (Kanatsu-Shinohara *et al.*, 2003; Hofmann et al., 2005; de Rooij, 2008).

Peritubular myoid cells and Sertoli cells have been reported to influence each other. Leydig cells can influence the Sertoli cell function through testosterone production because the Sertoli cells contain receptors for this hormone (de Rooij, 2009).

So the interplay of signalling factors coming from the different components of the SSC niche will direct the SSCs towards self-renewal or differentiation.

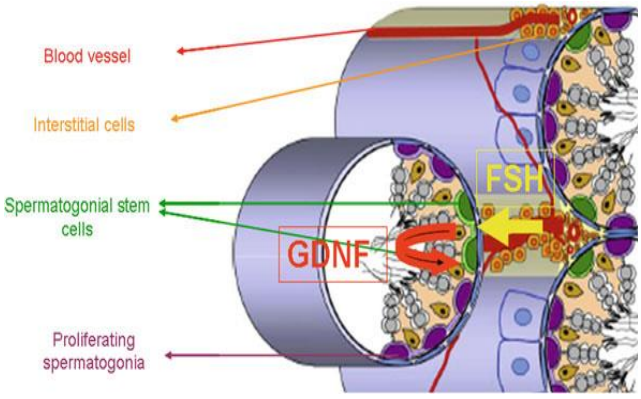

Figure 2. *Spermatogonial stem cell niche whereby Sertoli cells produce GDNF under stimulation of FSH (Goossens and Tournaye, 2013).*

1.2.2 Spermatogonial proliferation

The prevailing model of SSC proliferation in non-primates is the A_s-model (Huckins, 1971; Oakberg, 1971). The A_s or single undifferentiated type A-spermatogonia are considered to be the most primitive cells or true SSCs. A_s divide symmetrically to form two new stem cells, or two differentiated cells. When A_s spermatogonia divide and the daugther cells migrate seperately they will remain single stem cells. If the daughter cells divide incompletely and are connected by a cytoplasmic bridge, they will be destined for differentiation (de Rooij and van Beek, 2013). These cells are referred to as paired spermatogonia (A_{pr}). The division of paired spermatogonia results in four aligned spermatogonia (A_{al}), which are also connected to each other. Aligned spermatogonia divide a few times more leading to chains of 8, 16 or even 32 cells, and then they will undergo morphological changes and transform into differentiating A_1 spermatogonia (Kolasa *et al*, 2011). Differentiating spermatogonia undergo a few mitotic divisions with the formation of A_2, A_3, A_4, Intermediate and B spermatogonia (Figure 3). B spermatogonia will differentiate into spermatocytes (46 chromossomes; 4N), which will undergo meiosis (de Rooij and Russel, 2000; Aponte *et al*., 2005; de Rooij and Griswold, 2012).

In primates, two classes of A spermatogonia are present: A_{dark} spermatogonia, defined as reserve stem cells, and A_{pale} spermatogonia, which proliferate continuously producing B spermatogonia. A_{pale} spermatogonia can give rise to B spermatogonia and primary spermatocytes (Kolasa *et al.*, 2011)(Figure 3).

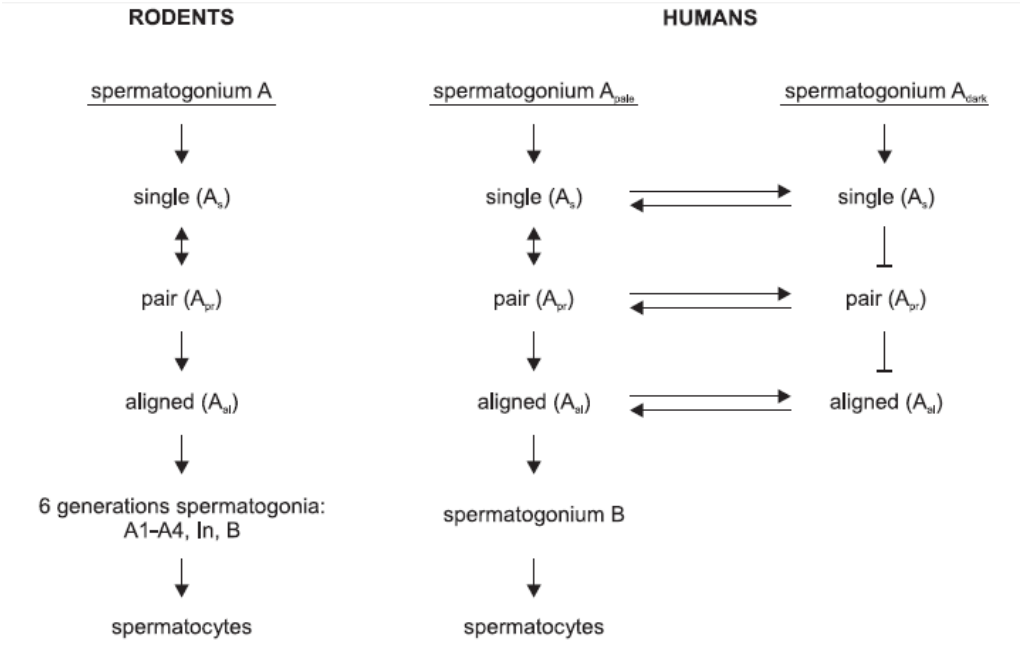

Figure 3. *Schematized spermatogonial stem cell renewal and multiplication in rodents and humans (Kolasa et al., 2011)*

1.2.3 Differentiation

Meiosis is the second step in spermatogenesis, in which primary spermatocytes undergo two divisions. The first meiotic division forms two secondary spermatocytes having 23 chromatides (2N). The second meiotic division gives rise to four haploid round spermatids with 22 autosomes (1N) and 1 sex chromosome (Figure 4).

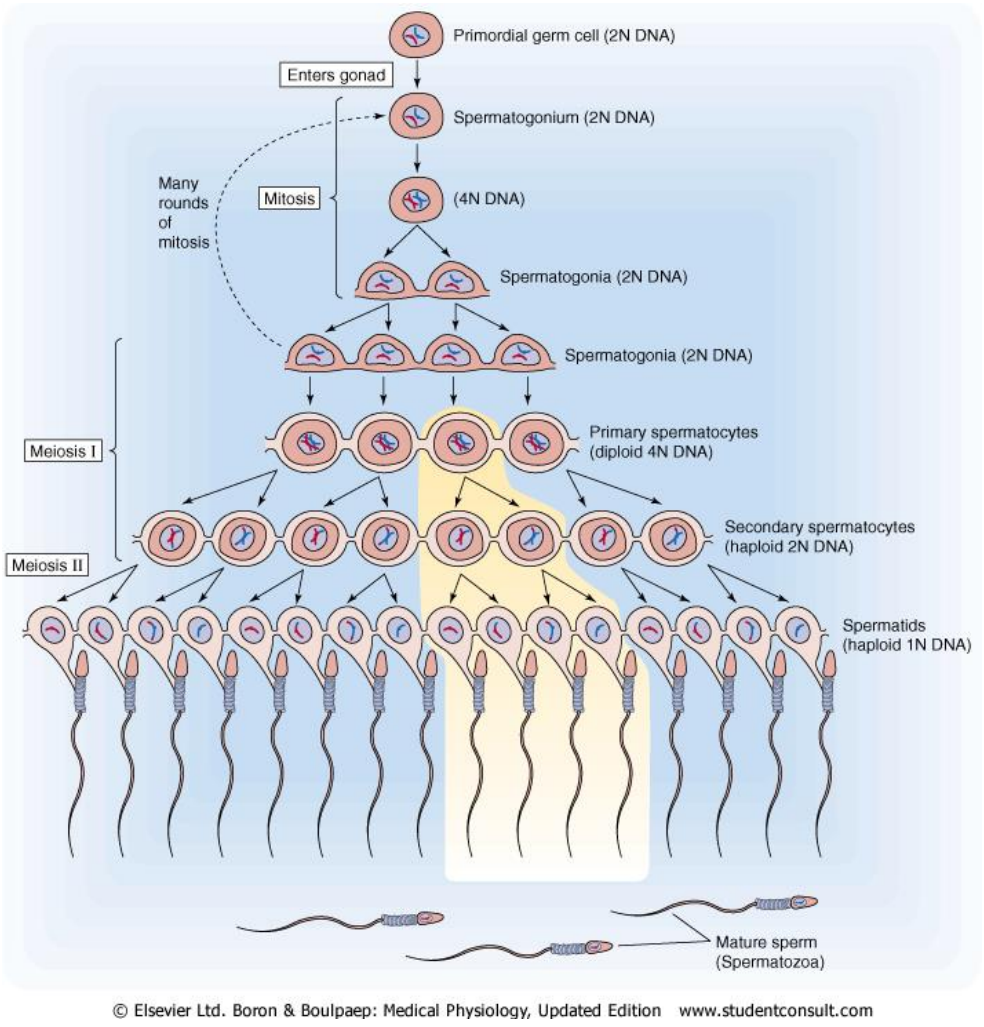

Figure 4. *Spermatogenesis.*

Once the spermatids are formed, they go through a few morphological changes characterized by condensation of the genetic material, the formation of the acrosome and the elongation of the tail to transform into spermatozoa in a process called spermiogenesis (Junqueira and Carneiro, 2004) (Figure 4).

The transformation of spermatogonia into a functional spermatozoon takes about 64 days in humans and around 35 days in the mouse (Brinster, 2007).

1.3 Epigenetics

'Epigenetics' refers to a collection of mechanisms and phenomena that can cause a change in the phenotype of a cell without altering its DNA sequence (Berger *et al.*, 2009). These epigenetic mechanisms involve DNA methylation, posttranslational histone modification, chromatin remodeling, and alterations in nuclear architecture (Figure 5) (Sims *et al.*, 2003; Gareth and Evan, 2009; Rajender *et al.*, 2011).

The best studied epigenetic changes are DNA and histone modifications, which occur during cell division and are transferred to the next generation. Other important epigenetic processes include genomic imprinting, gene silencing and X-chromosome inactivation. There are two main periods when cells undergo epigenetic reprogramming, namely during gametogenesis and early embryo development (Reik *et al.*, 2003). If there is an epigenetic mutation in the germ line, this can have adverse effects on the offspring. It is now well understood that a disrupted or altered epigenome can cause diseases (Feinberg, 2007).

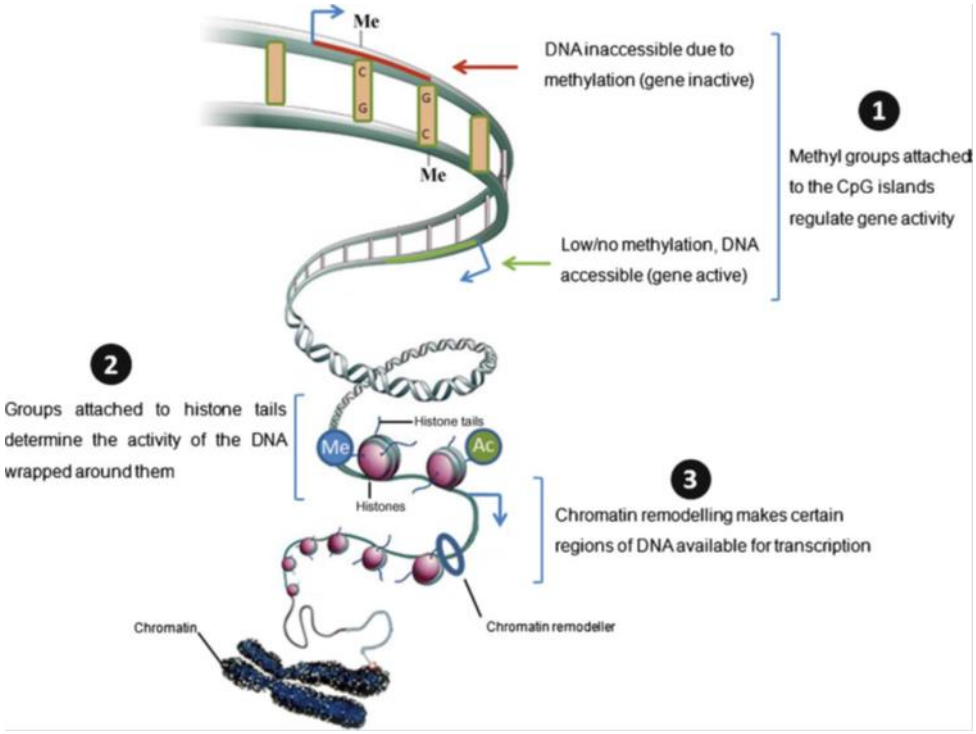

Figure 5. *Major epigenetic mechanisms affecting gene activity (Rajender et al., 2011).*

1.3.1 DNA methylation

DNA methylation is a heritable and reversible post synthetic DNA modification characterized by an enzymatic addition of a methyl group to the position of the adenine purine ring. The result is a physical blockade to transcriptional proteins and an affinity for methyl-CpG-binding domain proteins that form compact chromatin and silence genes (Chason *et al.*, 2011). In mammals DNA methylation occurs at the C5 position of cytosine (5-MC) (Cheng and Blumenthal, 2008).

The enzymes catalyzing methylation are divided into two categories: 'maintenance' and *de novo* DNA methyltransferases (DNMTs).

DNMTs can be classified in three groups: DNMT1 (DNMT1s, DNMT1o), DNMT2 and DNMT3 (DNMT3a, DNMT3b and DNMT3l). The DNMT3 family establishes the initial CpG methylation pattern *de novo* (Chen and Li, 2006), whereas DNMT1 has preference for hemi-methylated DNA and is critical for the maintenance of DNA methylation patterns in replicating cells. DNMT2 is a methyltransferase whose function is still unknown and controversial (Trasler *et al.*, 1992; Shi and Wu, 2009).

1.3.2 Histone modifications

Histones are highly conserved nuclear proteins that regulate DNA structural organization and gene expression (Tchurikov, 2005). Histones are rich in arginines and lysines that are subject to covalent modifications such as acetylation, methylation, phosphorylation, ubiquitylation… These modifications may cause gene activation or silencing (Palini *et al.*, 2011).

Depending on the arrangement of the histones with the DNA, the DNA appears as euchromatin, which is mainly transcriptionally active, or as heterochromatin, which is transcriptionaly silent (Grant, 2001; Govin *et al.*, 2004; Berger, 2007).

This thesis focusses on histone acetylation. During histone acetylation an acetyl group is transferred to the amino group of the lysine residue by the histone acetyltransferases. The charged portion of the histone is neutralized and its affinity to the DNA is mutually reduced. So histone acetylation facilitates the access of the RNA polymerases and transcription factors in the promoter region, leading to transcription.

Acetylated histones are mainly histone 3 (lysines 9, 14, 18 and 23) and histone 4 (lysines 5, 8, 12, and 16). Deacetylation of the histone reinforces the association with DNA and the transcription is suppressed (Grant, 2001).

1.3.3 Epigenetic modifications during spermatogenesis

Errors in the sperm's epigenetic information may cause infertility or may be transmitted to the next generation causing congenital defects in the offspring (Carrel & Hammoud, 2010). The genome of mature spermatozoa is highly methylated and this methylation pattern is obtained during gametogenesis (Reik and Dean, 2001). Establishment of the DNA methylation pattern occurs in spermatogonia and in spermatocytes, but it is not thought to happen in spermatids. In parallel, maintenance methylation has always been thought to take place in the context of DNA replication, therefore in mitotically dividing spermatogonia and in preleptotene spermatocytes (La Salle and Trasler, 2006).

De novo methylation begins in the male germ line before birth and is further consolidated after birth. DNMT3a is the predominant DNMT in differentiating spermatogonia, suggestive of a specific role for this isoform in these cells (La Salle and Trasler, 2006). A deficiency of this enzyme disrupts spermatogenesis and prevents the methylation of imprinted genes (Kaneda *et al.*, 2004). A null mutant lacking DNMT3a shows a reduced number of germ cells getting into meiosis during the initial spermatogenic wave, leading to the hypothesis that DNMT3a is important for the initiation of meiosis (Yaman and

Grandjean, 2006). Studies have shown that DNMT3a is expressed in type B spermatogonia and preleptotene spermatocytes (Watanabe *et al.*, 2004). DNMT3a expression is high in type A spermatogonia, slightly decreases in type B spermatogonia and reduces further in preleptotene spermatocytes. Expression in leptotene/zygotene spermatocytes is similar to that in type A spermatogonia. DNMT3a transcripts are also detected in round spermatids, but are at their lowest level in elongating spermatids (La Salle and Trasler, 2006).

During spermiogenesis, histones are replaced by protamines. Protamine - DNA interactions resulting in chromatin condensation is important for correct differentiation of round spermatids into mature spermatozoa. It is proposed that the histone acetylation is a preparatory step for the histone-to-protamine exchange. It is suggested that three general mechanisms act in combination to destabilize the nucleosomes and replace the histones: (1) large-scale incorporation of histone variants, creating less stable nucleosomes; (2) genome-wide histone hyper-acetylation; and (3) competition for DNA binding with very basic DNA-interaction non-histone proteins (Gaucher *et al.*, 2010).

While histone 4 acetylation occurs at the level of spermatogonia and preleptotene spermatocytes, it is absent in leptotene and pachytene spermatocytes. H4 is again acetylated in round spermatids. A too early acetylation of histone 4 will cause a premature condensation of the nuclei and will consequently lead to infertility (Sonnack *et al.*, 2002). The acetylation pattern on histones H4K5, H4K8, H4K12 and H4K16 in infertile men (round spermatid arrest) was different from fertile men (Hecht *et al.*, 2009). Although most of the histones are replaced by protamines, a certain fraction of histones remains present in mature spermatozoa.

1.3.4 Epigenetic modifications in pre-implatation embryos

Pre-implantation development is the period between oocyte fertilization and formation of the blastocyst. Pre-implantation development is characterized by extensive epigenetic modification of the newly formed embryonic genome, permitting the onset of a highly regulated gene expression program (Palini *et al.*, 2011).

Prior to fertilization, the genomes of both sperm and metaphase II oocytes are transcriptionally inactive. However, asymmetry exists in the chromatin structure of both gametes so that restructuring is required to form a functional embryonic nucleus before embryonic transcription and the tightly regulated post-fertilization developmental program of gene expression can be initiated (Young and Beaujean, 2004).

The newly formed zygote undergoes demethylation of the parental genomes in a chronological asymmetric manner (Shi and Wu, 2009). The paternal genome undergoes active demethylation followed by passive demethylation of the maternal genome (Chason *et al.*, 2011). The hypomethylated state is then gradually reversed in the morula stage by *de novo* methyltransferases (DNMT3a and DNMT3b), and coincides with initiation of cell differentiation (Figures 6) (Young and Beaujean, 2004; Chason *et al*, 2011).

Histone modifications are probably established during the blastocyst stage (Kim and Ogura, 2009). However, some histone marks are inherited from sperm. During

decondensation, the paternal genome becomes associated with hyperacetylated H4K8 and H4K12 (Palini *et al.*, 2011).

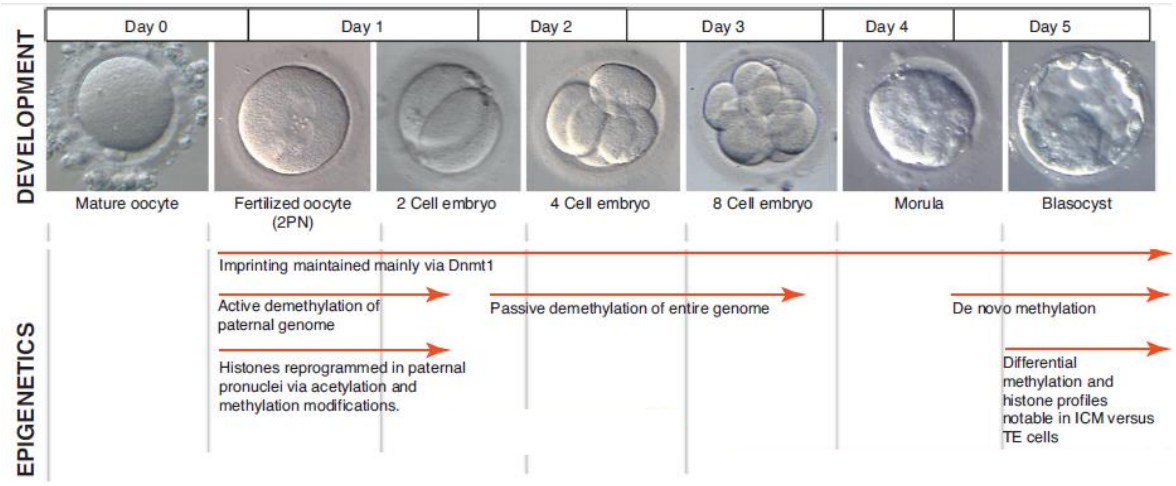

Figure 6. *Epigenetic changes during pre-implantation embryo development (Chason et al., 2011).*

1.4 Clinical problem

About one in every 600 children will develop cancer before the age of 15 years. However, due to the progress in the treatment of cancer, more than 80% of the children can now be cured (Tournaye *et al.*, 2004). At present, one in 1000 adults in the age group of 20-30 years old is a childhood cancer survivor (Hawkins and Stevens, 1996).

However, their future fertility may be compromised because of the malignancy itself, but more frequently, due to the treatment. A major side effect of radio and chemotherapy is the degeneration of spermatogenesis, resulting in infertility. Besides cancer, other diseases requiring gonadotoxic treatments (e.g. sickle cell disease) may lead to SSC loss in 80% of cases (Magelssen *et al.*, 2006). The testis has been shown to be highly susceptible to the toxic effects of these regimens (Rousseaux *et al.*, 1993; Monteil *et al.*, 1997; Robbins *et al.*, 1997; Jahnukainen *et al.*, 2011; Ortega and Tournaye, 2012). The prepubertal testis is extremely vulnerable due to its constant turnover of early germ cells and the maturation of the Leydig cell pool and other somatic compartments. Low-dose chemo- and/or radiotherapy can deplete (nearly) all progenitor type A_{pale} spermatogonia, differentiating B spermatogonia and spermatocytes in the adult testis, while in prepubertal testis, it partially eliminates or physiologically impairs the A_{dark} spermatogonia and the Sertoli cells (Figure 7). High doses of chemo- and/or radiotherapy cause more severe damage. All the SSCs commit to apoptosis and/or damaged Sertoli cells are unable to support the SSCs. This may lead to complete depletion of the SSC pool, resulting in seminiferous tubules with only Sertoli cells. In this case the patient becomes permanently infertile (Jahnukainen *et al.*, 2011).

Leukemia is the most common cancer in children, followed by cancers in the brain and the nervous system (Murk and Seli, 2012). Boys with acute leukemia requiring hematopoietic stem cell transplantation are at extremely high risk for testicular failure,

because the preparation for hematopoietic transplantation involves total body irradiation and/or high-dose chemotherapy (Jahnukainen *et al*., 2011).

Although the most important goal is to cure the patient from cancer, with the increasing survival rates amoung children, more attention should be paid to the quality of life after curement. Infertility can have a dramatic psychological and emotional impact on a person. Therefore, strategies have to be developed to preserve fertility in these patients. While for adult cancer patients, semen banking is the standard preventive strategy, the same is not true for prepubertal patients, because they lack active spermatogenesis (Tournaye *et al.*, 2004). Strategies for the prevention of treatment-induced male infertility in young boys implicate the preservation of SSCs before stem cell loss occurs. After treatment, the preserved SSCs could be re-introduced to the testis to re-establish spermatogenesis. The storage of prepubertal testicular tissue for future transplantation is currently offered by a few centres worldwide (Brinster, 2007).

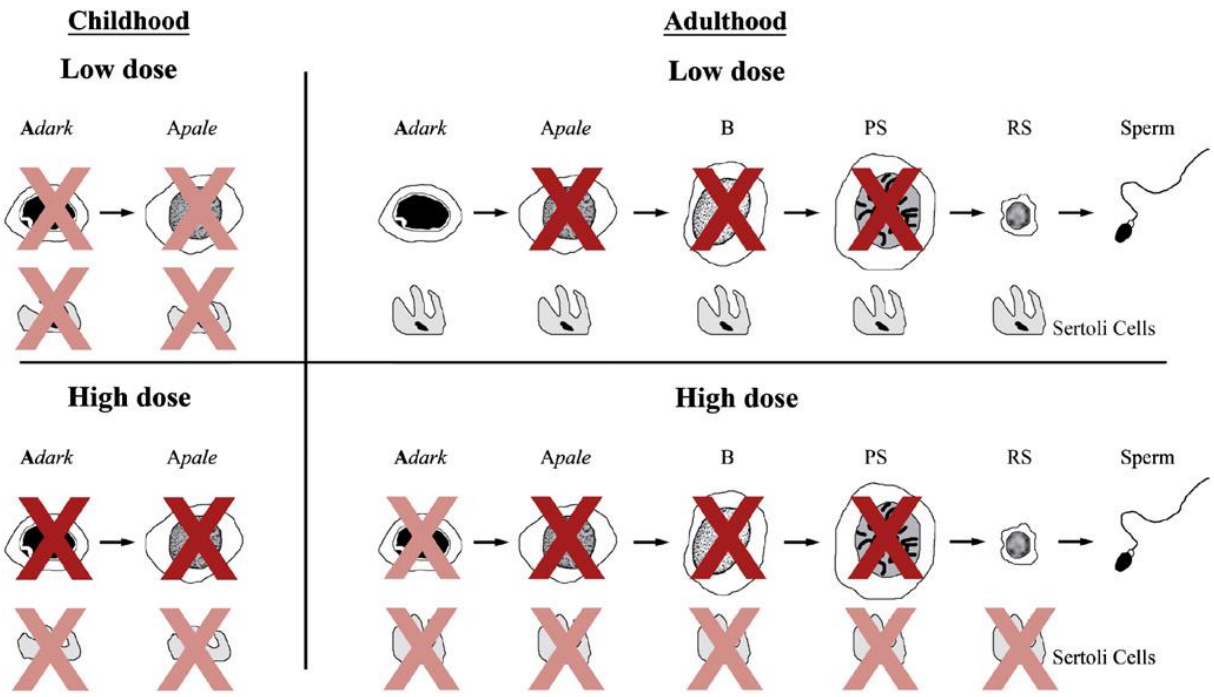

Figure 7. *Schematic illustration of the effects of low- and high-dose cancer treatments on germ and Sertoli cells in the seminiferous epithelia of primates during childhood and adulthood. Adark = A_{dark} spermatogonia; Apale = A_{pale} spermatogonia; B = B spermatogonia; PS = Pachytene spermatocytes; RS = Round spermatids. The dark red cross indicates a total or near total elimination of the corresponding cell type, while the light red cross indicates a partial elimination or physiological impairment (Jahnukainen et al., 2011).*

Almost 20 years ago, Brinster and colleagues introduced the transplantation of SSCs in mice (Brinster and Zimmermann, 1994). A germ cell suspension obtained from fertile donors was transplanted into infertile receptor mice by injection into the seminiferous tubules (Figure 8). The transplanted SSCs colonized the seminiferous tubules, started to proliferate and initiated spermatogenesis. Microscopic analyses showed that cell differentiation proceeded normally and that the cells showed a normal morphology. The

recipient mice were able to produce offspring carrying the donor genotype (Brinster and Avarbock, 1994).

To achieve efficient colonization of the SSCs at the basement membrane, it is important that recipient testes are devoid of endogenous spermatogenesis. However, depletion of endogenous germ cells needs to be achieved with a minimal damage to the local spermatogenic and systemic environment. The chemotherapeutic busulfan, which is a cytotoxic agent damaging spermatogenesis, is now generally used for the preparation of recipient mice for spermatogonial stem cell transplantation (SSCT) (Ogawa *et al*., 2000).

Full spermatogenesis has been reported after autologous transplantation in pigs, cattle and primates (Honaramooz *et al.*, 2002; Izadyar *et al.*, 2003; Hermann *et al.*, 2012)

In rodents and goats live births were obtained after natural mating (Avarbock *et al.*, 1996; Honaramooz *et al.*, 2003; Goossens *et al.*, 2006).

Even the transplantation between different species with close phylogeny was proven successful (Dobrinski *et al.*, 1999; Ogawa *et al.*, 1999).

Although fertility is restored in mice having undergone SSCT, we have still observed lower sperm concentrations and sperm motility compared with fertile controls (Goossens *et al.*, 2003 and 2008). In animals, healthy live offspring have been obtained after SSCT, displaying normal karyotypes and unmodified methylation levels in three investigated genes (Goossens *et al.,* 2009 and 2010). After transplantation, no significant abnormalities were found in levels of histone methylation and histone acetylation, except for H4K5ac and H4K8ac in late spermatocytes and round spermatids (Goossens *et al.*, 2011). Because these markers are important in elongated spermatids during the histone-protamine exchange it is important to perform more studies on the genetic and epigenetic level of the sperm and embryos obtained after transplantation.

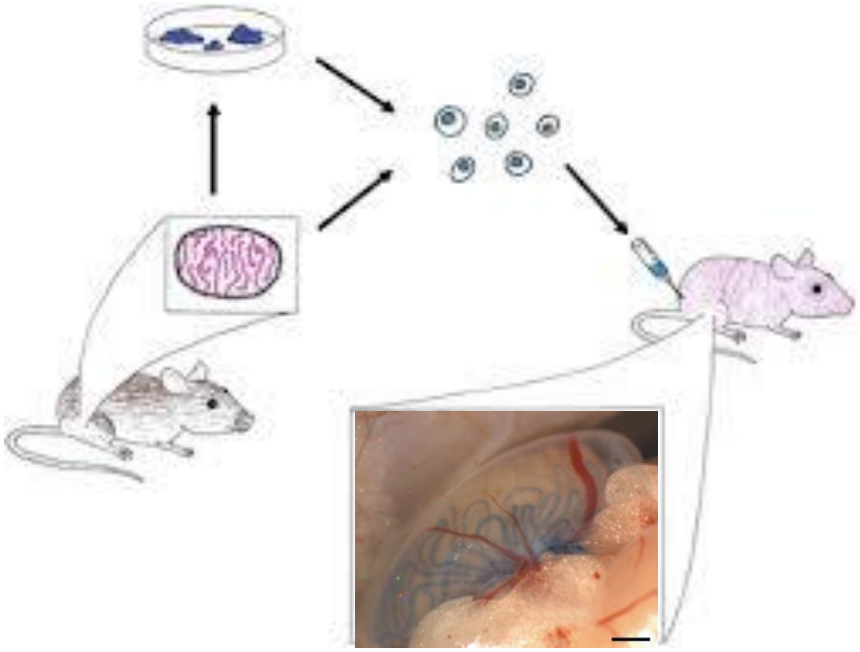

Figure 8. *Scheme of the spermatogonial stem cell transplantation: A germ cell suspension obtained from a fertile donor is transplanted into an infertile receptor mouse by injection into the seminiferous tubules.*
http://openi.nlm.nih.gov/imgs/rescaled512/2871929_rstb20100026-g2.png

1.5 Objectives

In previous studies, it was shown that no abnormalities occur at the genetic level after SSCT in the spermatozoa and in the born offspring (Goossens *et al.*, 2009 and 2010). Only the acetylation of H4K5 and H4k8, which is important for the histone-protamine exchange during spermiation, happened too early (in late spermatocytes and round spermatids) (Goossens *et al.*, 2011). A too early acetylation of H4K5 and H4K8 may lead to infertility. Because H4K8ac is hereditary through the paternal genome, the aim of the present project is to examine epigenetic modifications in embryos and offspring obtained after SSCT.

1. Evaluation of the general methylation level in pre-implantation embryos at different stages of development (2 and 4 cell stage, multi-cell, morula and blastocyst) using immunohistochemistry for 5-methylcytosin (5-MC).

2. Evaluation of H4K8ac in early embryos by immunostaining.

3. Evaluation of 5-MC, DNMT3a, H4K5ac and H4K8ac on testicular tissue from live born offspring obtained after SSCT.

2. Material and Methods

2. Material and Methods

2.1 Animals

The recipient mice for transplantation were obtained by crossing female inbred Sv129 green fluorescent protein (GFP^+) mice (gift from Whitehead Institute for biomedical research, Cambridge, MA, USA) with male inbred C57BL mice (Iffacredo, Brussels, Belgium). Male GFP^- F1-hybrids were used as recipients, while pre-pubertal GFP^+ F1-hybrids were used as donors.

All experimental procedures were approved by the ethical committee for animal experiments of the Vrije Universiteit Brussel.

2.2 Transplantation

The transplantations were performed last year by another master student.

Donor cells were obtained from six to ten days old males (SV129xC57Bl GFP^+ F1 hybrid) and the testes were collected in 2 ml Dulbecco's modified eagle's medium/F12 (DMEM/F12; 31330-038; Invitrogen, Merelbeke, Belgium). After decapsulation, the testes were digested according to a two-step enzymatic digestion protocol. First, the tissue was incubated in DMEM/F12 containing 1 mg/ml collagenase IV (C5138-1.G; Sigma) and 30 mg/ml deoxyribonuclease (DNAse; DN25; Sigma-Aldrich; Diegem; Belgium), in a warm water bath (37°C) for 15 minutes. The tissue was washed twice in 10 ml DMEM/F12 at 600 *g* for 5 minutes. Next, the cell pellet was incubated in DMEM/F12 containing 0.25% trypsin (T-9201; Sigma) and 1 mM ethylenediaminetetraacetic acid (EDTA; E-6511; Sigma), in a warm water bath (37°C) for 5 minutes.

Then, 4% of fetal calf serum (FCS; 10500-056; Invitrogen) and 30 mg/ml DNAse solution were added to stop the trypsin activity. The cell suspension was filtered through a nylon mesh with pore size 40 µm (352340; BD Biosciences, Erembodegem, Belgium) and centrifuged at 600 *g* during 5 minutes. After removal of the supernatant, 10 µl injection medium (DMEM/F12 with 5% penicillin streptomycin (P/S; P4333; Sigma) and 5% FCS) was added per mg tissue to get a concentration of 10 to 20 x 10^6 cells/ml. The cell suspension was kept at 4°C until transplantation.

Twelve week old SV129xC57Bl GFP^- F1 hybrid mice were used as recipients. These mice were sterilized by i.p. injection with the chemotherapeutical Busulfan (40mg/Kg). They were anesthetized with a mixture of 0.1 mg/ml Medetor® (Pfizer Animal Health NV, Louvain-La-Neuve, Belgium) and 0.75 mg/ml Ketamine® (Santé Animale, Libourne, France), dissolved in saline. A dose of 75 µl/10 g body weight of the anesthetic mixture was injected i.p. Recipient transplantations were performed under a stereomicroscope. To prevent hypothermia of the animal, mice were kept on a heated (37°C) platform during transplantation.

After disinfection of the incision area with Hibitane® (703158; Two Omega Drive, Manchester, England), the abdomen was opened and the testes were exteriorized.

Hereafter, the thin membrane between the testis and the epididymis was cut, the efferent duct was immobilized and the pipette was introduced in the rete testis via the efferent duct.

In the tip of the pipette, trypan blue dye was added to visualize the entry of the injected cells in the seminiferous tubules (Ogawa *et al*., 1997).

Immediately after transplantation the mice were injected s.c. with 100 µl antibiotics [100 µl Baytril® 2.5 % (Bayer, Diegem, Belgium) + 1900 µl physiological solution].

2.3 Testicular histology of the transplanted males

More than one year after transplantation, recipient testes were collected in 2 ml DMEM/F12. The tunica albuginea was removed and the testicular tissue was fixed in Hidrosafe (10056580; Labonord, Rekkem, Belgium) for at least 1 hour. Fixed tissue was embedded in paraffin, and 5µm thick sections were made and stained with hematoxylin and eosin to analyze the structure of the tubules. After deparaffinization in xylene and rehydratation in 100%, 100%, 90% and 70% isopropanol, sections were incubated for 3 minutes in hematoxylin, dipped in acid alcohol and Li_2CO_3 before being incubated in eosin for 1 minute. The sections were dehydrated in a mounting series of alcohol (70%, 90%, 100% and 100%) and finally in xylene and mounted using acrytol mounting medium (100406; Surgipath, Labonord, Rekkem, Belgium).

The sections were also stained for GFP to confirm the success of the transplantation. The sections were deparaffinized in xylene for 10 minutes (three times) and rehydrated in a descending alcohol series. Sections were washed for 5 min in phosphate buffered saline (PBS). Endogenous peroxidases were blocked with 0.3% H_2O_2 for 30 minutes. Afterwards, sections were washed in PBS for 5 min, and then 3% of normal goat serum (NGS) was added to the sections for blocking non-specific binding for 30 minutes. The primary antibody (1/100; sc-9996; Bioconnect, Huissen, Nederland) was added and incubated overnight at 4°C. GFP+ tissue without adding primary antibody was used as negative control. For the negative controls no primary antibody was added. The next day, sections were washed three times with PBS after which the secondary antibody was added for one hour at room temperature. The sections were again washed three times with PBS. The visualization of the staining was done with DAB. The preparations were immersed in PBS and counterstained with hematoxylin. The sections were dehydrated in a mounting series of alcohol and finally in xylene. To quantify the results, the total number of seminiferous tubules with full and normal GFP^+ spermatogenesis and the number of tubules with no GFP^+ spermatogenesis was assessed. The sections were analyzed under an Olympus IX 81 inverted microscope using bright field.

2.4 *In vivo* conception

To get the test embryos, the transplanted males were crossed with SV129 x C57Bl GFP^- females. Negative control embryos were obtained by mating GFP^- males and GFP^- females. Positive control embryos were obtained by crossing GFP^+ males with GFP^+ females. Males older than 8 weeks were mated with females of 3 to 5 weeks.

Three to five weeks old female (C57BL X Sv129) mice were stimulated through an i.p. injection (Figure 9) with 5IU pregnant mare's serum gonadotropin (PMSG; A023A02, Intervet, Brussels, Belgium), followed by an injection with 5IU human chorionic gonadotropin (hCG; A030A01, Intervet) 46-48h later. Two females were caged with one male immediately after the administration of hCG.

Figure 9. *I.p. injection in a mouse*

2.5 Embryo collection and fixation

Embryos were collected at different stages (2-cell, 4-cell, multi-cell, morula and blastocyst) as described previously (Hogan *et al*., 1994). Females were sacrificed by cervical dislocation. Dissection of the reproductive organs of the female mice was performed to collect the embryos. The two-cell and four-cell embryos were collected between 30-52 hours post-hCG from the oviduct. Morula embryos were collected from the oviduct 70-75 hours after hCG injection. Finally, balstocysts were collected 94-100 hours post-hCG from the uterus (Figure 10). Specific medium was used for each embryonic stage: cleavage medium (G20720, K-SICM-20, SYDNEY IVF, Australia) for 2-cell, 4-cell and morula stages; and blastocyst medium (G20722, K-SIBM-20, SYDNEY IVF, Australia) for blastocysts.

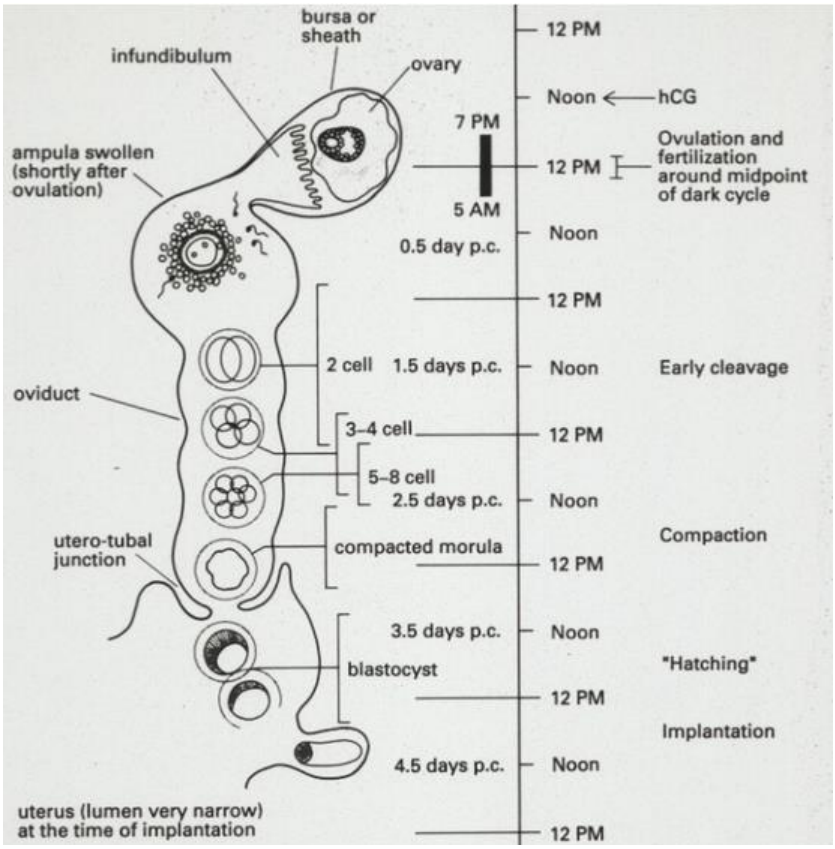

Figure 10. *Scheme of pre-implantation development (Hogan, 1994)*

Embryos were fixed in 96 well-plates with wash solution [PBS + 2% bovine serum albumin (BSA)] and fixative (PBS + 3.7% formaldehyde). The embryos were shortly washed and then transferred to 50 µl fixation drops for 10 minutes. Hereafter the embryos were washed three times for 5 minutes each. The embryos were stored in 100 µl PBS with 2% BSA at 4 °C.

2.6 Embryo stainings

2.6.1 H4K8ac double staining

After washing the embryos shortly in 50 µl of wash solution, they were transferred into permeabilization droplets (50 µL PBS + 0.1% Triton X - 100) during 20 minutes. After washing, a serum blocking step was performed (10% NGS) for 30 minutes at room temperature. Before incubation with the primary antibodies, there were two washing steps, one short and one for 5 minutes. The embryos were incubated with the first antibodies overnight at 4°C. To distinguish the GFP^+ embryos, a double staining was performed with GFP antibody (sc-9996; Bioconnect) together with the H4K8ac antibody (ab15823; Abcam, Cambridge, UK). The droplet with the isotype control contained rabbit IgG (sc-2027; Santa Cruz Biotechnology, Heidelberg, Germany) (Table I).

The next day, the embryos were washed three times (one short washing step and two for 5 minutes) before incubation with the secondary antibodies, goat anti-rabbit (Alexa Fluor 647; Invitrogen) for H4K8ac and goat anti-mouse (Alexa Fluor 488; Invitrogen) for GFP (Table I), for 2 hours at 4°C in the dark. Afterwards, the embryos were washed again 3 times (one short and two for 5 minutes) and then transferred into a droplet of Slow fade (Gold antifade Reagent with DAPI; S36939; Invitrogen) between two glass coverslips (24 - 50 nm) and preserved in the dark at 4°C.

2.6.2 5-MC double staining

After washing the embryos shortly in 50 µl of wash solution, they were transferred into permeabilization droplets (50 µl PBS + 0.3 % Triton X - 100) for 30 minutes. After permeabilization the embryos were washed in washing solution during 5 minutes followed by a wash step in PBS for 5 minutes. Hereafter, the embryos were denatured in 6M HCl during 10 minutes. Then, the embryos were washed shortly in PBS, followed by four washing steps of 5 minutes with washing solution. To distinguish the GFP^+ embryos a double staining was performed with GFP antibody (ab6658; Abcam) together with the 5-MC antibody (NA81; Calbiochem, Darmstadt, Germany) (Table I). The well with the isotype control contained mouse IgG (MCA928; Abd Serotec, Düsseldorf, Germany). The embryos were incubated with the primary antibodies overnight at 4°C.

Next day, the embryos were washed three times (one short and two of 5 minutes) before incubation with the secondary antibodies, donkey anti-mouse (Alexa Fluor 647; Invitrogen) for 5-MC and donkey anti-goat (Alexa Fluor 488; Invitrogen) for GFP (Table I), for 2 hours at 4°C in the dark. Afterwards the embryos were washed again three times (one

short and two of 5 minutes) and then transferred to a droplet of Slow fade between two glass coverslips (24 - 50 nm) and preserved in the dark at 4°C.

Table I Conditions for immune staining of the embryos.

1st Antibody	Dilution factor	2nd Antibody	Dilution factor	Isotype control	Dilution factor
H4K8ac	1/100	Goat anti-rabbit; Goat anti-mouse	1/200	Rabbit IgG	1/100
GFP	1/200				
5-MC	1/500	Donkey anti-mouse; Donkey anti-goat	1/200	Mouse IgG	1/50
GFP	1/200				

2.7 Testicular histology of the offspring born after SSCT

Right after transplantation, mice were caged together with 2 GFP- females each to evaluate their fertility and the success of transplantation with the birth of GFP^+ offspring. Testicular tissue was obtained from 16 week-old GFP^+ offspring of transplanted mice. The mice were sacrificed by cervical dislocation and the testes were collected in 2 ml DMEM/F12. The tunica albuginea was removed and testicular tissue was fixed in Hidrosafe (10056580; Labonord) for at least one hour. After embedding in paraffin, the testicular tissue was cut into slices of 5 µm.

The sections were deparaffinized in xylene (X-Solv; Yvsolab, Beerse, Belgium) for 10 minutes (three times) and rehydrated in a descending alcohol series (100%, 100%, 90% and 70% isopropanol). Sections were washed for 5 min in PBS. For 5-MC (NA81; Calbiochem, Darmstadt, Germany), DNMT3a (sc-20703; Santa Cruz Biotechnology), H4K5ac (ab51997; Abcam), H4K8ac (ab15823; Abcam), the blocking of endogenous peroxidases (30 minutes) preceded the antigen retrieval step. After each step, sections were washed in PBS for 5 min. NGS was added to the sections for blocking non-specific binding. The specific primary antibodies were added and incubated overnight at 4°C. For the negative controls no primary antibody was added (Table II).

The next day, sections were washed three times with PBS after which the second antibody was added for one hour at room temperature. The sections were again washed three times with PBS. The visualization of the staining was done with DAB (Dako Envision Kit; K500711; Heverlee, Belgium). The preparations were immersed in PBS and counterstained with hematoxylin. The sections were dehydrated in a mounting series of alcohol and finally in xylene. They were mounted using acrytol mounting medium.

Table II Conditions for imuno staining of the testicular tissue

Antibody	Dilution factor	Antigen retrieval	Blocking endogenous peroxidades	Blocking non-specific binding
DNMT3a	1/250	Citrate buffer for 10 min (microwave: 350W)	3% H_2O_2	4% NGS
5-MC	1/200	Citrate buffer for 75 min (waterbath 95°C)	6% H_2O_2	10% NGS
H4K5ac	1/1500			5% NGS
H4K8ac	1/1250			

A series of consecutive histological sections was stained with the above mentioned antibodies. Each tubular cross section was scored for stage (according to Ventela *et al.*, 2002; Table III) and antibody positivity in the different levels. For each spermatogenic stage, the localization of the epigenetic marker was reported as well as the percentage of the tubules in which the marker was expressed. The sections were analyzed under an Olympus IX 81 inverted microscope using bright field.

Tabela III The XII stages of the seminiferous epithelial cycle in the adult mouse testis.

Stages	I-IV	V-VI	VII-VIII	IX	X-XI	XII
Level 3 (at the lumen)	Elongated spt	Elongated spt	Elongated spt			
Level 2	Round spt	Round spt	Round spt	Round spt	Round spt	Elongated spt
Level 1	Pachytene spc	Pachytene spc	Pachytene spc	Pachytene spc	Pachytene and diplotene spc	Second meiotic division
Level 0 (at the basement membrane)	Intermediate spg	B-spg	Preleptotene spc	Leptotene spc	Leptotene and zygotene spc	Zygotene spc

Spt, spermatid; spc, spermatocyte; spg, spermatogonia.

2.8 Statistical analysis

All data were analyzed by the Chi-square test to compare the differences in the epigenetic markers (5-MC, DNMT3a, H4K5ac and H4K8ac) during spermatogenesis between the offspring of the transplanted males and the controls. Significant difference was considered when $P < 0.05$.

3. Results

3. Results

3.1 Transplanted males

Last year, a master student performed 24 transplantations and analyzed 12. The other 12 were analyzed this year. After mating with females, GFP^+ pups were generated by two transplanted males (mouse 11 and 12). Immunohistochemical analysis for GFP^+ on 88 sections (4 sections for each testis) revealed spermatogenesis in only one of the two transplanted males who were able to produce GFP^+ pups. The other male with GFP^+ pups had a tumor on the place of the right testis. GFP^+ spermatogenesis was also observed in two transplanted males who did not father GFP^+ pups. In all the remaining transplanted males, no GFP^+ spermatogenesis was observed (Figure 11; Table IV).

Table IV Fertility assessment in transplanted males

Mouse ID	Success of Transplantations	GFP^+ spermatogenesis (%)	GFP^+ pups
1	R OK	0	0
	L OK	0	
2	R NOT	0	0
	L OK	0	
3	R OK	0	0
	L OK	**11**	
4	R NOT	0	0
	L OK	0	
5	R NOT	0	0
	L OK	0	
6	R OK	No testis found	0
	L NOT	0	
7	R OK	0	0
	L OK	**3**	
8	R OK	0	0
	L NOT	0	
9	R OK	0	0
	L NOT	0	
10	R OK	0	0
	L NOT	0	
11	R OK	**36**	**45**
	L OK	**30**	
12	R NOT	Tumor	**12**
	L OK	0	

R, right testis; L, left testis; OK, successful transplantation; NOT, no transplantation.

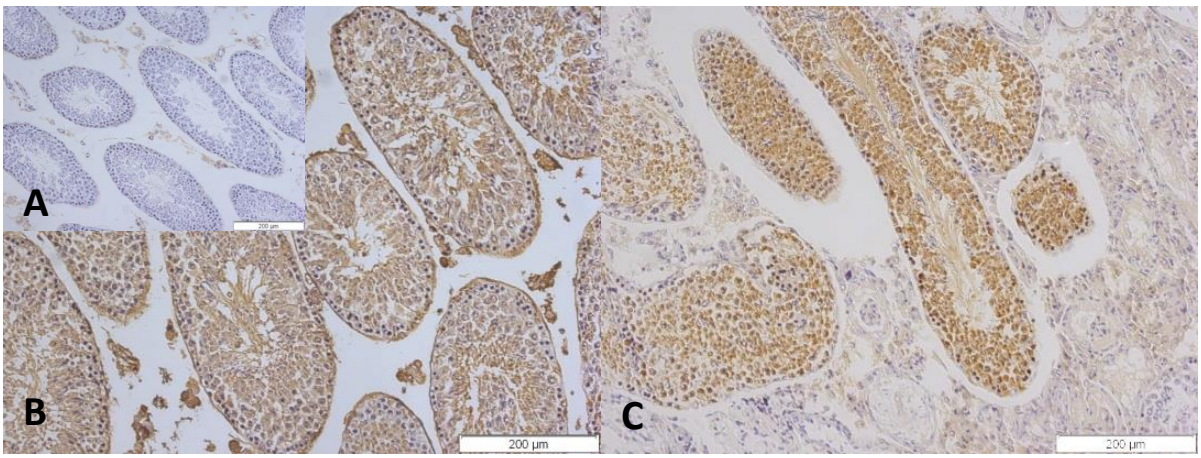

Figure 11 . *Immunohistochemical analysis of the expression of GFP. Representative adult control (A) negative control tissue (insert), and transplanted male (C). Cells expressing the marker stain brown..*

3.2 Embryos

The two transplanted males who were able to produce GFP^+ offspring were used for the *in vivo* conception experiment. In total, 260 embryos were collected from females mated with transplanted mouse 11, and 157 from mating with transplanted mouse 12.

Forty-six embryos were lost while performing the H4K8ac and 5-MC stainings. A total of 371 embryos were stained and evaluated (Table V). Unfortunately, all embryos were GFP^- (Figure 12 and 13). The positive controls showed positive staining for H4K8ac and 5-MC where expected.

Tabela V **Number of embryos stained for each development stage**

Development stage	Number of embryos
2-cell	184
4-cell	45
Multi-cell	36
Morula	60
Blastocyt	46
Total	**371**

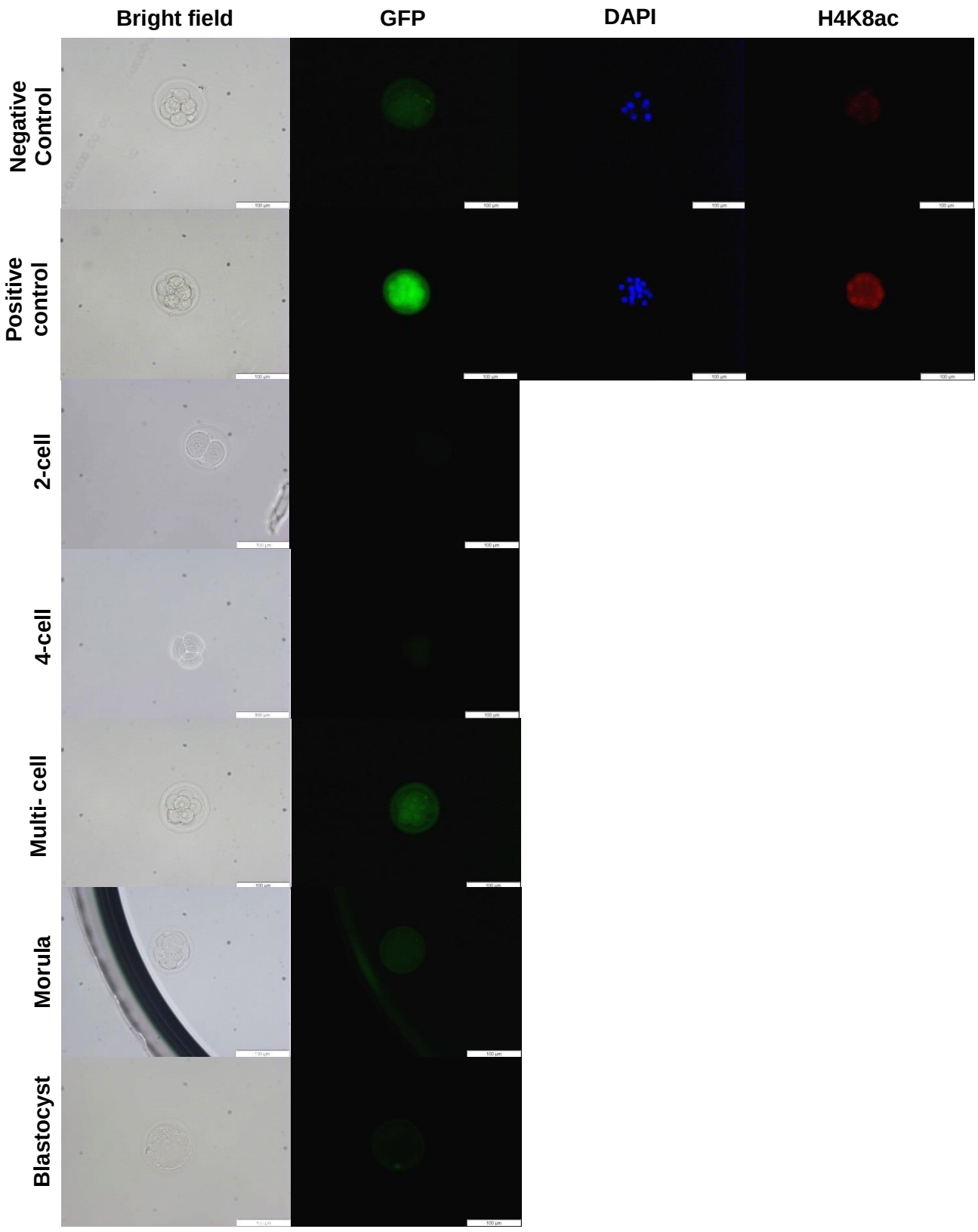

Figure 12 . *Expression and localization of H4K8ac in multi-cell stage of development on controls and expression of GFP on test embryos in all stages of development*

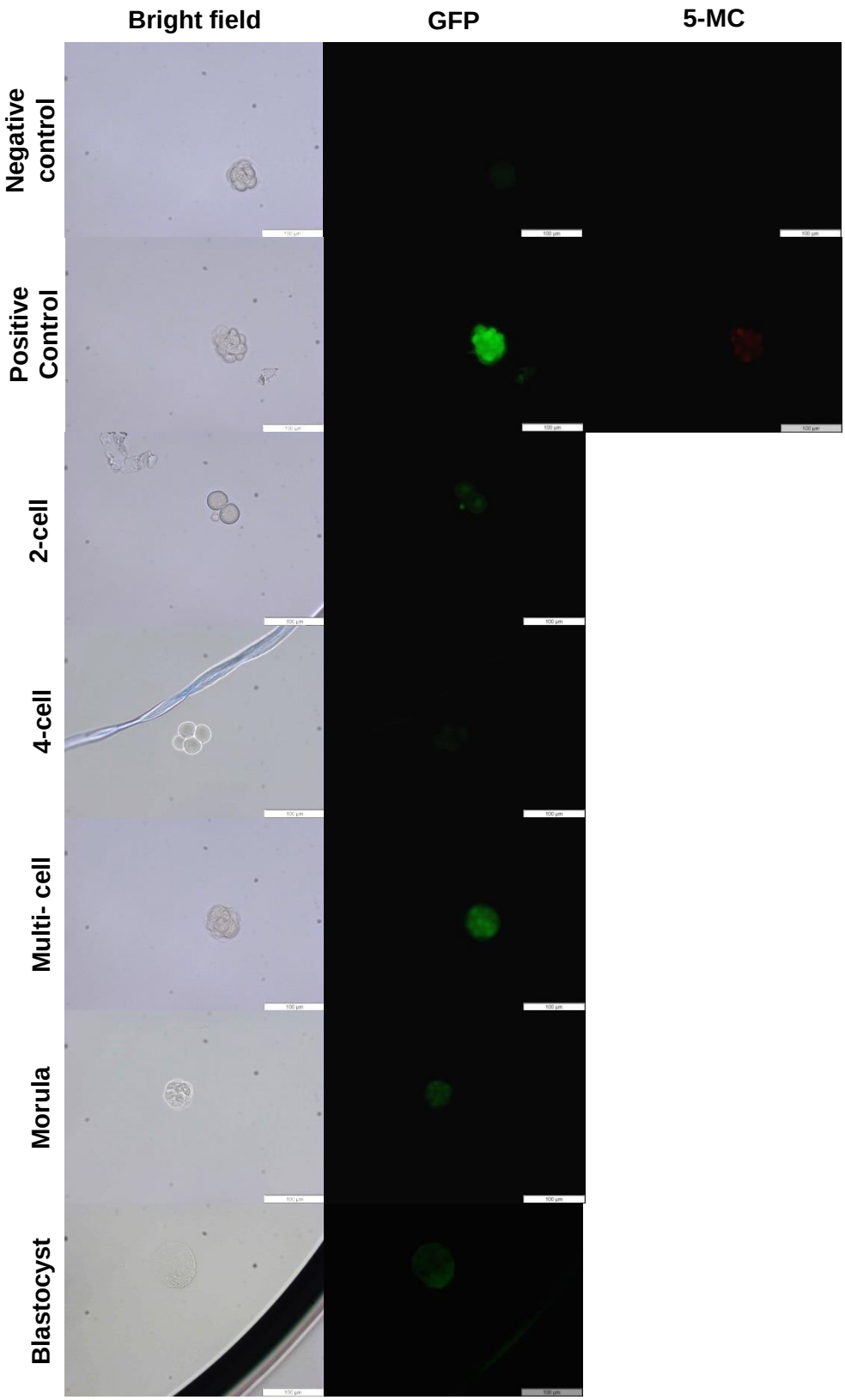

Figure 13 . *Expression and localization of 5-MC and GFP in multi-cell stage on controls and expression of GFP in test embryos in all stages of development*

3.3 Offspring

The testes of 23 GFP$^+$ offspring, obtained from four different transplanted males, were evaluated. Two of the transplanted males were sacrificed and analyzed by last year's master student. For each of the four stainings, 184 testicular tissue sections of the offspring were evaluated.

3.3.1 General methylation status

In control tissue, 5-MC expression was observed from spermatogonia up to zygotene spermatocytes and in round spermatids. The same expression pattern was observed in the offspring except for the expression of 5-MC in intermediate spermatogonia ($p < 0.05$) which was less observed in the test group (Figure 14). In control mice, methylation was observed in spermatogonia at stages I–IV for all tubules (100%), but in the SSCT offspring only 22% of the tubules were stained (Table VI).

Table VI Expression of the general methylation status of germ cells in control adult tissue and tissue of offspring obtained after SSCT.

5-MC		I-IV	V-VI	VII-VIII	IX	X-XI	XII
Control	level 3						
	level 2				100	100	NO
	level 1						
	level 0	100	15	100	100	100	NO
Offspring	level 3						
	level 2				94	100	98
	level 1						
	level 0	22*	9	100	100	100	98

0-20%
21-40%
41-60%
61-80%
81-100%

For each spermatogenic stage, the percentage of tubules expressing the marker was determined. Statistical differences are shown by asterisks. NO – Not observed

The testicular tissue of SSCT offspring showed a methylation in zygotene spermatocytes and elongated spermatids (stage XII), while in the control it could not be observed, because no tubules were scored at stage XII of spermatogenesis.

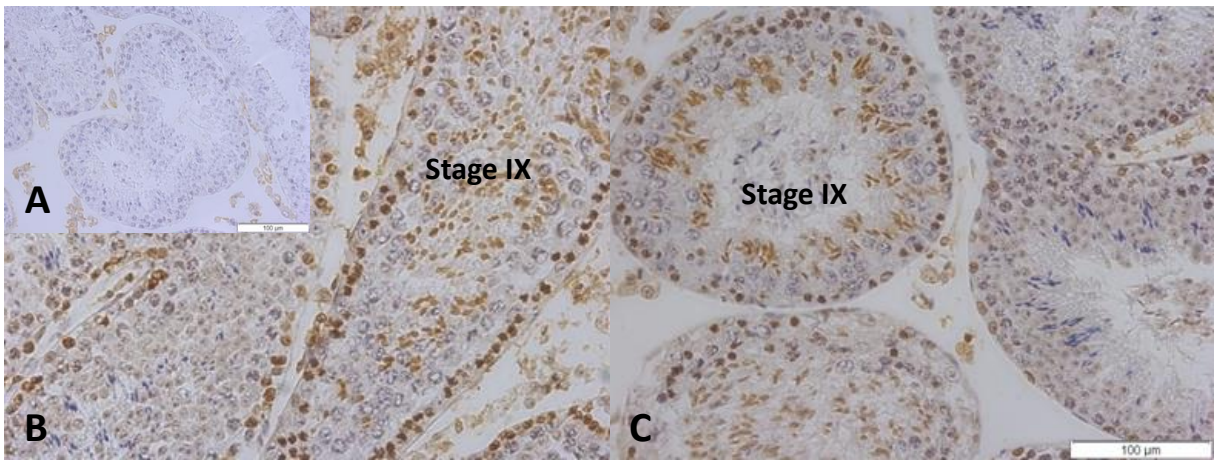

Figure 14. *Immunohistochemical analysis of the expression of 5-MC. Negative control (A), representative adult control (B), the offspring tissue examined (C). Cells expressing the marker stain brown. The tubular stage is indicated.*

3.3.2 DNMT3a expression

DNMT3a is a *de novo* methyltransferase that ensures the installation of a new methylation pattern. In both the positive control and offspring obtained after SSCT, DNMT3a expression was only detected in spermatogonia and (pre)leptotene spermatocytes (Figure 15). No differences were observed between controls and offspring (Table VII).

Table VII Expression of DNMT3a in germ cells in control adult tissue and tissue of offspring obtained after SSCT.

DNMT3A

		I-IV	V-VI	VII-VIII	IX	X-XI	XII
Control	level 3						
	level 2						
	level 1						
	level 0		100	100	100		
Offspring	level 3						
	level 2						
	level 1						
	level 0		81	100	90		

0-20%
21-40%
41-60%
61-80%
81-100%

For each spermatogenic stage, the percentage of tubules expressing the marker was determined. Statistical differences are shown by asterisks.

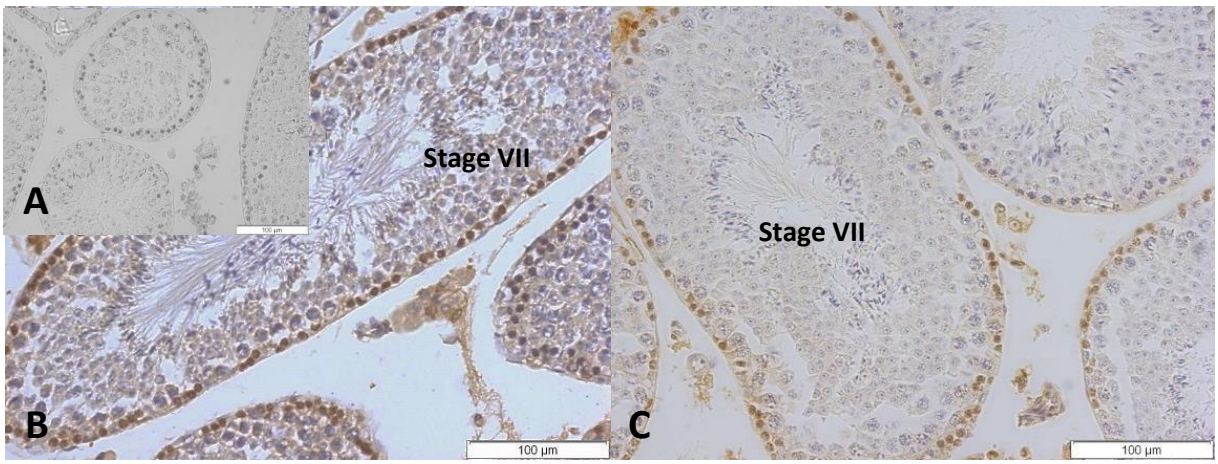

Figure 15. *Immunohistochemical analysis of the expression of DNMT3a. Negative control (A), representative adult control (B), the offspring tissue examined (C). Cells expressing the marker stain brown. The tubular stage is indicated.*

3.3.3 Histone modifications

The acetylation of lysines 5 and 8 on histone 4 is important especially during the histone-protamine exchange in elongated spermatids.

In the adult control, all spermatogonia were stained for H4K5ac in tubules at stage I–VI. All preleptotene spermatocytes were also stained (Table VIII; Figure 16). Furthermore, H4K5ac was vastly detected in round and elongated spermatids at stage IX–XII. In the offspring born after SSCT, positive staining was additionally found in pachytene spermatocytes of some tubules in stage VII-VIII ($p < 0.05$).

Table VIII H4K5ac staining in germ cells in control adult tissue and tissue of offspring obtained after SSCT.

H4K5ac		I-IV	V-VI	VII-VIII	IX	X-XI	XII
Control	level 3						
	level 2				100	100	100
	level 1	90	80				
	level 0	100	100	100	100	100	90
Offspring	level 3						
	level 2				100	100	100
	level 1	78	78	7*			
	level 0	100	100	100	87	83	83

0-20%
21-40%
41-60%
61-80%
81-100%

For each spermatogenic stage, the percentage of tubules expressing the marker was determined. Statistical differences are shown by asterisks.

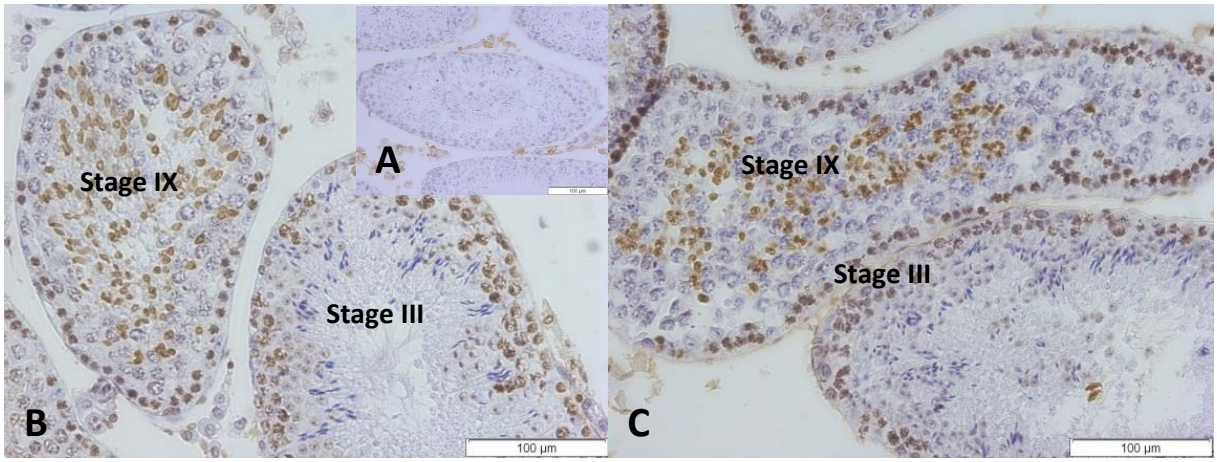

Figure 16. *Immunohistochemical analysis of the expression of H4K5ac. Negative control (A), representative adult control (B), the offspring tissue examined (C). Cells expressing the marker stain brown. The tubular stage is indicated.*

While in the control group no staining was observed in the leptotene and zygotene spermatocytes (stages IX-XII), in the offspring after SSCT H4K8 was acetylated ($p<0.05$) (Figure 17). There is also a significant difference between the control group and the offspring when it comes to the elongated spermatids in stage XII ($p<0.01$) (Table IX). Intermediate spermatogonia are stained in the group of the offspring (20%), but this was not the case in the control group ($p<0.05$).

Table IX **H4K8ac staining in germ cells in control adult tissue and tissue of offspring obtained after SSCT.**

H4K8ac

		I-IV	V-VI	VII-VIII	IX	X-XI	XII
Control	level 3						
	level 2				90	95	100
	level 1						
	level 0			73			
Offspring	level 3						
	level 2	2			100	100	4*
	level 1						
	level 0	20*		100	45*	45*	20*

0-20%
21-40%
41-60%
61-80%
81-100%

For each spermatogenic stage, the percentage of tubules expressing the marker was determined. Statistical differences are shown by asterisks.

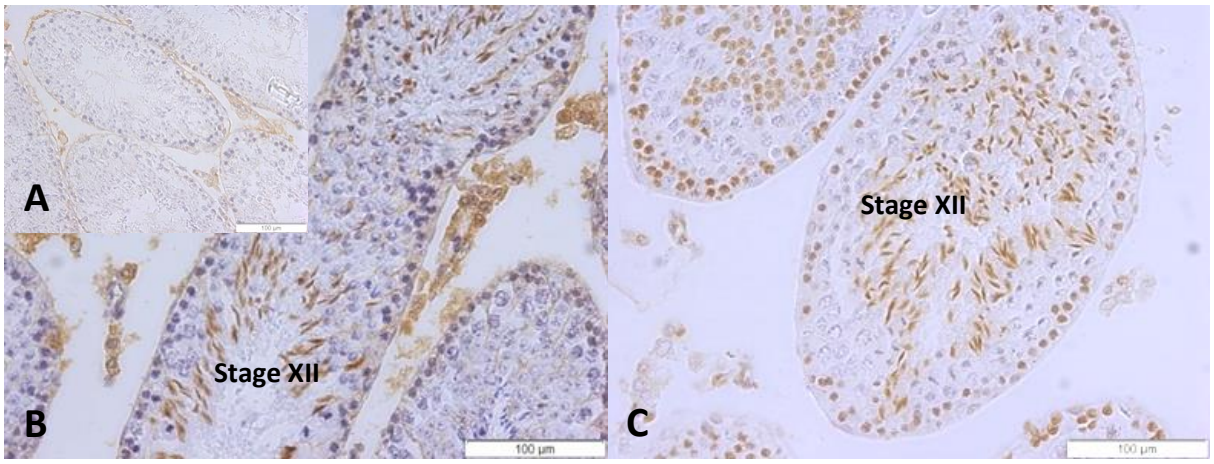

Figure 17 . *Immunohistochemical analysis of the expression of H4K8ac. Negative control (A), representative adult control (B), the offspring tissue examined (C).Cells expressing the marker stain brown. The tubular stage is indicated.*

4. Discussion

4. Discussion

The technique of spermatogonial stem cell transplantation was developed in 1994 (Brinster and Avarbock, 1994; Brinster and Zimmerman, 1994). It has been shown that there were no chromosomal and genetic changes detectable after SSCT in mice (Goossens *et al.*, 2010). However, differences were found on the acetylation of H4K5 and H4K8 in round and early elongated spermatids (Goossens *et al.*, 2011). Nowadays more and more clinical centers start to invest in a cryopreservation programme for prepubertal testicular tissue as a fertility preservation strategy for boys facing SSC loss.

But, before SSCT can be performed clinically, all safety concerns must be evaluated. Histone acetylation plays an important role during spermatogenesis since infertile men present a different pattern of acetylation on H4K5, H4K8, H4K12 and H4K16 (Hecht *et al.*, 2009). It is also known that H4K8ac is hereditary from sperm to embryo. Transmission of genetic or epigenetic errors to embryos might lead to abnormal embryonic development and anomalies after birth.

Therefore, the current study was designed to check epigenetic modifications in preimplantation embryos and offspring obtained after SSCT.

In the study of Brinster and Avarbock, 71 % of the injected testes had a successful initiation of spermatogenesis between 3 and 10 months after transplantation. Also in the study of Goossens *et al* (2003), the efficiency of successful initiation of spermatogenesis was high (64%) after 5 months of transplantation. In our study, males were sacrificed more than one year after transplantation. From 12 transplanted males only 3 presented tubules with normal GFP^+ spermatogenesis. From these 3, only one had GFP^+ pups. The other two were not able to produce progeny, probably because the amount of full GFP^+ spermatogenesis was too low: 11% and 3%. Anyway, out of 152 pups obtained from male 11, only 45 were GFP^+ and 12 out of 36 pups from male 12 were GFP^+. This low efficiency may be explained by the fact the transplantations had been performed by a master student, who was not yet familiar with the technique. The technique of SSCT is difficult and has its learning curve.

In these experiments a testis cell suspension, which was not enriched for SSCs was injected. Enrichment for SSCs can improve the outcome of the transplantation. The efficiency of SSCT can thus be improved by isolation of stem cells by Fluorescent-Activated Cell Sorting (FACS) and/or Magnetic-Activated Cell Sorting (MACS), or expansion of SSCs *in vitro* to increase the number of SSCs prior to transplantation, and thus the success of re-establishing spermatogenesis (Kanatsu-Shinohara *et al*., 2003).

Unfortunately, out of 371 embryos evaluated none were GFP^+. This might have happened due to bad luck of timing. All embryos were derived from endogenous spermatogenesis (GFP^-). At the time of mating, the tubules with donor spermatogenesis (GFP^+) might have been in an early stage of spermatogenesis. Another explanation is that an error has been made during the staining procedure. However this is not likely since the positive controls did stain for GFP. Most probably, the fact that the embryos, fertilized with GFP^+ sperm, were heterozygotes and the controls were homozygotes caused this

discrepancy. The controls might have a stronger expression of GFP, which is more easily detectable under the microscope. In the future, heterozygote controls should be used to ensure that there is no difference of GFP expression under the microscope.

We expected donor spermatogenesis in the left testis from male 12, since this male obtained GFP^+ pups. However no GFP^+ spermatogenesis was found. So maybe the successful transplantation was performed on the right testis and not on the left. Unfortunately, we could not evaluate the right testis because of a tumor. If the GFP^+ pups were obtained from spermatozoa developed in this right testis, it may explain why the male stopped having GFP^+ pups since the experiment started and why no GFP^+ embryos could be obtained.

A correct DNA methylation is also important during spermatogenesis since it is important for the maintenance of the embryo integrity and transmission of the epigenetic information to the next generation.

Less spermatogonia were stained for 5-MC in the offspring, but they were stained as expected in the controls. This difference could be explained by a counting error, with some tubules not very clearly stained. The leptotene and zygotene spermatocytes and spermatids were positively stained in offspring as well as in the controls. Unfortunately, in the controls, there were no tubules at stage XII to compare with. During meiosis, in the leptotene/zygotene stage, the DNA replication is completed and the maintenance methylation initiated. We did not observe methylation in pachytene spermatocytes and round spermatids, but it is expected that, there too, methylation is present (Oakes *et al.*, 2007). This may be explained by the fact that during meiosis there is a 'dilution' of methylation since each pachytene spermatocyte has one methylated strain and another one still unmethylated. The primary spermatocytes have two methylated strands.

DNMT3a is a *de novo* methyltransferase, indispensable for the methylation of DNA. DNMT3a is the predominant DNMT in differentiating spermatogonia, suggesting a specific role for this isoform in these cells (La Salle and Trasler, 2006). DNMT3a is expressed in B spermatogonia and preleptotene/leptotene spermatocytes from stage V to stage IX in both the control and the test group. Our findings are in agreement with the studies by Watanabe *et al* (2004) and Goossens *et al* (2011).

However in the study of La Salle and Trasler (2006), DNMT3a expression is high in type A spermatogonia, slightly decreases in type B spermatogonia and reduces further in preleptotene spermatocytes. Expression in leptotene/zygotene spermatocytes is similar to that in type A spermatogonia. DNMT3a transcripts are also detected in round spermatids, but are at their lowest level in elongating spermatids. These results were obtained using real-time RT-PCR to determine the expression levels of the DNMT gene in total RNA extracted from all cell types. The different method of evaluation may explain the observed differences. Maybe, both techniques, histochemistry and RT- PCR, should be used to compare and find more certain results.

The offspring born after SSCT were evaluated for histone modifications H4K5ac and H4K8ac. These markers are important for the histone-to-protamine exchange during spermatogenesis (in the elongated spermatids). Histone-to-protamine exchange in haploid spermatids is preceded by hyperacetylation of core histones resulting in decreased DNA-histone interaction. H4 acetylation is associated with active transcription in early spermatogenesis and in round spermatids. H4 should be acetylated in mouse spermatogonia and preleptotene spermatocytes, deacetylated throughout meiosis and in round spermatids, and then re-acetylated in elongating spermatids (Hazzouri *et al.*, 2000). The acetylation of H4K5 and H4K8 in the offspring was similar to the control group. The only difference was observed in the pachytene spermatocytes (stage VII-VIII), which were not stained in the control group, but showed some acetylation in the test group. The study of Sonnack *et al* (2002) reported that an additional signal in the spermatocytes in the seminiferous tubules of men with round spermatid maturation arrest indicates that early hyperacetylation of histone H4 may lead to premature nuclear protein transitioning and subsequent infertility. This needs further investigation.

For acetylation of H4K8, we did not observe any staining in the leptotene and zygotene spermatocytes in the control groups, but in the SSCT offspring acetylation was observed. A too early acetylation of histone 4 might cause premature condensation of the nuclei and may consequently lead to infertility (Sonnack *et al.*, 2002). The acetylation of H4K5 and H4K8 was normal in the elongated spermatids.

Although H4K5ac and H4K8ac differed in some aspects from the controls, the H4 acetylation presented correctly during the histone - protamine exchange in the elongated spermatids.

In conclusion, no main differences were observed in 5-MC, DNMT3a and H4K5ac expression. Although we detected abnormal acetylation of H4K8 in leptotene and zygotene spermatocytes in the SSCT offspring, the acetylation of H4K8 was normal in the elongated spermatids, at the time the histone-to-protamine exchange takes place. However, previous studies have shown that the offspring grew up normally and were able to have progeny theirselves (Goossens *et al.*, 2009). Therefore the abnormalities observed do not seem to cause severe disorders or infertility, at least in mice.

Transplantation of cryopreserved testis cells isolated from cancer patients carries an inherent risk of reintroducing contaminating malignant cells back to the patients (Pacey 2007; Jahnukainen *et al*., 2011). In some studies, FACS has been shown to remove cancer cells from testis cell suspensions of leukemic mice (Fujita *el al*, 2005), but others do not confirm total removal (Geens *et al*, 2007, Hermann *et al*, 2011). Most recent, Dovey *et al* (2013) demonstrated that a multiparameter analysis and sorting strategy can enrich spermatogonia and eliminate cancer contamination from a human testis cell suspension.

In the future, it would be necessary to repeat the embryo experiment to check possible epigenetic problems during embryo development. Therefore, a higher number of successfully transplanted males should be used to collect embryos. Heterozygotes controls should be also used to check if there's a difference on the expression of GFP under the microscope.

SSCT might become a powerful tool to restore fertility in patients suffering from stem cell loss. At this moment the transplantation of SSCs is still at an experimental phase, but the results obtained so far are promising. Therefore testicular tissue banking may be advised as a fertility preservation strategy for prepubertal boys facing gonadotoxic treatments (Tournaye *et al*., 2004; Goossens and Tournaye, 2013).

We conclude that SSCT has potential as a clinical application, but more research is required before becoming a fertility preservation technique.

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